Human Kallikrein-6 (Neurosin) ELISA Kit General Protocol

FIVEphoton Biochemicals

For research use only.
Not for diagnostics.

Part No. hKLK6-ELISA

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This copy is provided for reference. Use the protocol shipped with your kit for your experiment.

Store: 4°C. Expiration: 6 months after arrival.
Safety: Stop solution contains acid. Avoid all contact and inhalation. Wear eye protection.

Kallikrein-6 ELISA Kit

Part No. hKLK6-ELISA

For research only. Not for diagnostic applications in humans.

Storage: 4°C, for up to six months after arrival.

Safety: Stop solution contains acid. Avoid eye and skin contact

Standard Peptide: 90 ng/ml

Assay Range: 2 - 70 ng/ml

Experimental Principles

This kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to measure Kallikrein-6 concentration. Kallikrein-6 is a soluble secretory protein[1], and therefore located in intracellular organelles of the secretory pathway (i.e. endoplasmic reticulum, Golgi, endosomes, etc), and in the extracellular space due to secretion. It can be extracted from organelles by using a detergent protein extraction reagent compatible with ELISA, such as a Triton based - HEPES buffer[2], or assayed directly in cell culture media, serum and plasma. If assaying cell culture media, a protein concentrator, such as a centricon, may be employed to meet the assay concentration range.

To measure Kallikrein-6 concentration, samples are applied in the provided microelisa plate. This plate has wells pre-coated with an affinity purified polyclonal anti-human Kallikrein-6 antibody. The samples are first incubated with the antibody-coated plate, and then washed. An anti- Kallikrein-HRP conjugate antibody is then added, followed by an incubation period, and subsequent wash. Chromogen solutions A and B are applied resulting in a blue coloration change. A stop solution is added to terminate the reaction, turning the solution to yellow. Absorbance readings at 450 nm and correspondence to standard peptide concentrations are employed to determine Kallikrein-6 concentration in the samples.

The KLK-6 ELISA kit detects Kallikrein-6 in a variety of sources, including serum, CSF, plasma, cell culture supernatant, tissue homogenates and cell lysates. Prepare the samples as described below.

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1. Tatebe H, Watanabe Y, Kasai T, Mizuno T, et al. 2010. Extracellular neurosin degrades α-synuclein in cultured cells. *Neurosci Res*. Aug;67(4):341-6.
2. Fivephoton Biochemicals Part no. ELSP-1 or similar.

Generic Sample Preparation Suggestions: Use 50 ml culture tubes and a desktop cell culture centrifuge for the following procedures. Your experiment may require other methods for sample preparation not discussed here.

1. **Serum**: Coagulate at room temperature for 10-20 min. Centrifuge for 20 min at 2000-3000 rpm. Collect the supernatant; if precipitation appears, centrifuge again. Assay the supernatant fraction.
2. **Plasma:** Use suitable EDTA or heparin as an anticoagulant. Mix for 10-20 min using a stir bar. Centrifuge for 20 min at 2000-3000 rpm. Collect the supernatant; if precipitation appears, centrifuge again.
3. **Urine**: Collect in a sterile container. Centrifuge 20-min at 2000-3000 rpm. Collect supernatant; if precipitation appears, centrifuge again. .

4. **Cell culture supernatant:**. Detection of secretory components: Centrifuge culture media for 20 min at 2000-3000 rpm to sediment cells. Collect supernatant. Use a protein concentrator if needed.
5. **Cell cytoplasm:** Dilute cell suspension with PBS (pH7.2-7.4) to a cell concentration of 1 million cells / ml. Perform repeated freeze-thaw cycles to fracture the cell membrane and to release intracellular components. Centrifuge for 20min at 2000-3000 rpm. Collect supernatant for your assay; if precipitation appears, centrifuge again. A detergent cell lysis buffer can also be used to collect a soluble supernatant fraction for ELISA assay.
6. **Cellular organelles and membranes**. Use a detergent protein extraction reagent compatible with ELISA. Lyze cells and dissolve membranes. Collect the supernatant for further measurements.
7. **Tissue:** Cut and weigh tissue slice. Use a detergent protein extraction buffer such as FIVEphoton Biochemicals Part No. TEB-1 and the recommended protocol to extract KLK-6. Perform ELISA on the supernatant fraction.
8. Samples can be store at -80^{o}C. Avoid repeated freeze-thaw cycles. You may aliquot samples for later ELISA assays.
9. **Avoid denaturing cell lysis buffers that contain SDS, such as RIPA buffer.**

Table 1. Materials supplied. Store all materials at 4^{o}C

1	Standard peptide 90ng/ml	0.5ml		7	Chromogen Solution A	6ml
2	Standard diluent	1.5 ml		8	Chromogen Solution B	6ml
3	Microelisa Strip plate	12 well×8strips		9	Stop Solution	6ml
4	HRP-Conjugate Antibody	6 ml		10	Instruction Manual	1
5	30×wash solution	20ml		11	Sealed bags	1
6	Sample Diluent	6ml				

Materials required but not supplied

1. 37^{o}C incubator
2. Standard absorbance microplate reader
3. Precision pipettes and disposable pipette tips
4. Deionized water
5. Disposable tubes for sample dilution
6. Absorbent paper
7. 96 well dishes for preparation of solutions prior to transfer to the ELISA dish
8. 96-channel transfer pipette

Important notes and preparation for the assay

1. It is recommended that the experimenter perform preliminary tests to identify the sample concentration that meets the assay range. Perform a preliminary assay with samples using standards at the high and low dilution. Suspend and dilute experimental samples into the “Sample Diluent (Table 1, Component 6)” to meet the assay range, (alternatively, dilute samples in PBS with a protein blocking reagent; e.g. 0.5% Casein). A dilution series of several samples may be required to identify the correct sample concentration that meets the assay range. Concentrate or dilute experimental samples if adjustments are needed. Set aside sufficient experimental samples as a reserve to repeat the assay.
2. Determine whether the vehicle buffer inadvertently cross-reacts with the assay and generates a color change by performing vehicle only controls. Additionally, determine whether ingredients in the vehicle buffer inhibit the assay;

dilute the provided standard peptide in the vehicle and perform an assay test. Compare results to the same standard peptide dilution in the Sample Diluent (Table 1, Component 6). For remedy, dilute samples in "Sample Diluent" (Table 1, Component 6) or prepare samples in another vehicle (such as PBS with protein blocker) to prevent inadvertent experimental readings or assay inactivation.

3. The kit should be equilibrated to room temperate for 30 min prior to performing the assay. Store opened microelisa plates in a sealed plastic bag at 4°C. A multi-channel pipettor is recommended to apply samples simultaneously. Plates should be sealed during the assay. Wells should not be allowed to dry.
4. Prepare standards and samples in separate tubes or 96-well plates, not in the ELISA plate wells. Transfer standards and samples simultaneously to the ELISA plate.
5. It is recommended that samples are assayed in duplicate to address pipetting error.
6. Use new applicator tips and ELISA plate sealants to avoid cross-contamination.
7. Do not mix reagents from other ELISA kits.
8. Note that sodium azide in samples that is not washed away may inhibit HRP.
9. When calculating concentration of your sample, make sure to take into account the dilution factors.
10. If the wash solution crystallizes during storage at 4°C, heat solution at 37°C and shake until crystals suspend.

Assay procedures

Standard and Sample Preparation. Standards and Samples should be added simultaneously to wells**. Prepare the standards and samples in a separate 96-well dish and transfer simultaneously to the ELISA dish.**

Assay procedure

1. Set aside and mark 12 wells for standard peptide dilutions. Configure six concentrations of standard peptide in duplicate as indicated in Table 2 below. Do not use the ELISA wells directly to perform the dilutions. The final total volume in each well should be 50µl

Table 2. Standard Dilutions

Well	Standard Concentration	Standard Number	Dilution Instructions
1	60 ng/ml	1	Mix 100µl Standard Peptide (Table 1, Component 1) with 50µl Standard Diluent (Table 1, Component 2). Remove 100µl to make standard 3.
2	60 ng/ml	2	Mix 100µl Standard Peptide with 50µl Standard Diluent. Remove 100µl to make standard 4.
3	40 ng/ml	3	Mix 100µl Standard Number 1 with 50µl Standard Diluent. Remove 100µl to make standard 5.
4	40 ng/ml	4	Mix 100µl Standard Number 2 with 50µl Standard Diluent. Remove 100µl to make standard 6.
5	20 ng/ml	5	Mix 100µl Standard Number 3 with100µl Standard Diluent. Remove 100µl to make standard 7. Remove 50µl, discard.
6	20 ng/ml	6	Mix 100µl Standard Number 4 with100µl Standard Diluent. Remove 100µl to make standard 8. Remove 50µl, discard.
7	10 ng/ml	7	Mix 100µl Standard Number 5 with 100µl Standard Diluent. Remove 100µl to make standard 9. Remove 50µl, discard.
8	10 ng/ml	8	Mix 100µl Standard Number 6 with 100µl Standard Diluent. Remove 100µl to make standard 10. Remove 50µl, discard.
9	5 ng/ml	9	Mix 100µl Standard Number 7 with 100µl Standard Diluent. Remove 50µl to make standard 11. Remove 100µl, discard.
10	5 ng/ml	10	Mix 100µl Standard Number 8 with 100µl Standard Diluent. Remove 50µl to make standard 12. Remove 100µl, discard.
11	2.5 ng/ml	11	Mix 50µl Standard Number 9 with 50µl Standard Diluent. Remove 50µl to make Standard
12	2.5 ng/ml	12	Mix 50µl Standard Number 10 with 50µl Standard Diluent. Remove 50µl to make Standard 12.

2. Set up 2 blank wells separately. In blank wells, add 40μl of the provided Sample Diluent (Component 6) and 10μl vehicle buffer that the sample is in; do not add sample. Perform all other procedures of the assay, except omit the HRP-conjugate antibody (Table 1, Component 4).

3. For wells with experimental samples, add 40μl provided Sample Diluent for each well, then add 10μl of experimental sample. This creates a 5X dilution factor of sample which should be accounted for later when calculating sample concentration. This preparation should be done in separate tubes or 96-well plates, and not in the ELISA dish.

4. Transfer the standard peptide solutions, blank well solutions and diluted experimental samples simultaneously to the ELISA dish. Use the closure membrane to enclose the plate, mix gently with a rotator table, and incubate for 30 min at 37°C, or 1 hr at RT. Dilute the wash solution during incubation as described in step 5.

5. Dilute the 30X Wash Solution (Table 1, Component 5) with dH_20. Make 3 ml of diluted wash solution for each assay well.

6. After the first incubation period, discard the liquid in the wells by gently aspirating. Turn the plate upside down and gently pat dry the plate with absorbent paper. To wash the wells, simultaneously fill each well with 100 μl of diluted Wash Solution, oscillate gently with the rotator table for 30 sec, and then aspirate off liquid. Pat dry the ELISA microplate with absorbent paper. Repeat the wash steps 5 times.

7. For each assay well, prepare in a separate 96-well dish 50 μl HRP-conjugate reagent (Table 1, Component 4), except the blank wells. Simultaneously transfer 50 μl HRP-conjugate reagent to each well, except for blank wells. Rotate gently for 1 hr at 37°C for 30 min or 1 hr at RT.

8. Wash the wells 5X as described above in step 6. Remove liquid from the wells. Do not allow wells to dry.

9. In a separate 96-well plate, mix 50 μl Chromogen Solution A (Table 1, Component 7) with 50μl of Chromogen Solution B (Table 1, Component 8) for each well. Transfer the chromogen mixture to each well. Mix the ELISA plate gently for 15 min at 37°C in the dark.

10. Simultaneously add 50 μl of Stop Solution (Table 1, Component 9) to each well. Upon addition of stop solution, the blue color should immediately change to yellow.

11. Measure the optimal density (OD) at 450 nm within 15 minutes after adding stop solution. Set the blank wells as zero.

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Data Analysis

1. Compile a standard curve using the blank standard solutions and corresponding OD values. You may wish to

calculate a linear regression equation to determine the concentration of your samples. Remember that samples were diluted 5 fold in the assay in your final calculation. Other data analysis methods to calculate sample concentrations are also applicable.

Flow chart of the procedures

Prepare standards, blank and samples

Add samples to wells, incubate for 1 hr at RT or 30 min at 37^{o}C.

Wash each well five times.

Add HRP-conjugate antibody to each well, incubate for 1 hr at RT or 30 min at 37^{o}C.

Wash each well five times

Add chromogen solutions A and B, 15 min at 37^{o}C, dark

Add stop solution

Measure OD 450 nm within 15 min